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SHANKS & HERBERT

Intellectual Property
Attorneys at Law

Of Counsel:

Patricia Ann Johnson
David W. Woodward

TransPotomac Plaza
1033 N. Fairfax Street
Suite 306
Alexandria, VA 22314

Telephone: 703-683-3600
Facsimile: 703-683-9875
Internet:
Toni@mail.ipadvocates.com

October 26, 2000

Box Patent Application

Assistant Commissioner for Patents
Washington, DC 20231

Re: New U.S. Patent Application
Atty. Docket No.: 0217-0004

Sir:

The following documents are forwarded herewith for appropriate action by the U.S.
Patent and Trademark Office:

1. U.S. Utility Patent Application entitled: rpoB Gene Fragments and a Method for the Diagnosis and Identification of Mycobacterium Tuberculosis and Non-Tuberculosis Mycobacterial Strains

claiming priority to: Republic of Korea Patent Application No. 1999-46795, filed October 27, 1999;

and naming as inventor(s):

Hyeyoung LEE

Address - No.190-1106, Woosung APT., Yangjae-1-dong, Seocho-ku,
Seoul, Republic of Korea,
Citizenship - Republic of Korea

Yong Kil PARK

Address - 534-1, Seongnam-dong, Joonwon-ku, Seongnam-si, Kyunggi-do, Republic of Korea
Citizenship - Republic of Korea

Gill-Han BAI

Address - No. 129-805 Parktown, Naejeong-dong, Boondang-ku,
Seongnam-si, Kyunggi-do, Republic of Korea
Citizenship - Republic of Korea

Sang-Jae KIM

Address – No. 310-103, Seonsoochon APT., 89, Banglee-dong,
Songpa-ku, Seoul, Republic of Korea

Citizenship – Republic of Korea

Sang-Nae CHO

Address – No. 111-602, Hansin APT., 929,
Mok-6-dong, Yangchon-ku, Seoul, Republic of Korea

Citizenship – Republic of Korea

Yeun KIM

Address – 56-10, Moonsan-ri, Moonsan-eup, Pajoo-si,
Kyunggi-do, Republic of Korea

Citizenship – Republic of Korea

Hee Jung PARK

Address – No. 102-209, Dongjin-villa, 341, Chang-1-dong,
Dobong-ku, Seoul, Republic of Korea

Citizenship – Republic of Korea

the application comprising:

a. A specification containing:

(i) **28** pages of description prior to the claims;

(ii) **1** page of claims (**5** claims);

(iii) a one (1) page abstract;

b. **10** sheets of drawings: (Figures 1a-7c);

2. Submission of Sequence Listing Under 37 C.F.R. § 1.821(a);
3. 8 pages of Sequence Listing and electronic copy on diskette (in PatentIn format);
4. An original, executed Combined Declaration and Power of Attorney;
5. Recordation Cover Sheet;
6. An original, executed Assignment;

7. An original, executed Verified Statement (Declaration) Claiming Small Entity Status – Small Business Concern;
8. Certified copy of priority document;
9. Check in the amount of \$395 (\$355 filing fee and \$40 recordation fee); and
10. Two (2) return postcards.

It is respectfully requested that, of the two attached postcards, one be stamped with the filing date of these documents and returned to our courier, and the other prepaid postcard be stamped with the filing date and unofficial serial number and returned as soon as possible.

If the Filing Fee is found to be insufficient for any reason, please charge such deficiency to our Deposit Account No. 50-0622.

Sincerely,



Toni-Junell Herbert
Reg. No. 34,348

Date: 10/26/00

Shanks & Herbert
TransPotomac Plaza
1033 N. Fairfax Street, Suite 306
Alexandria, VA 22314
(703) 683-3600

VERIFIED STATEMENT (DECLARATION CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(c)) - SMALL BUSINESS CONCERN

Docket No.: _____

In re the Application or Patent of:

Serial or Patent No:

Filed:

For:

J0917 U.S. PRO
09/697123
10/27/00

I hereby declare that I am

- ☒ the owner of the small business concern identified below:
☐ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: ERUME BIOTECH CO., LTD.

ADDRESS OF CONCERN: 4th Fl. Erume Building, 807-3, Bangbae-dong, Seocho-ku, Seoul
137-060, Republic of Korea

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 37 C.F.R. § 1.9(d), for purposes of paying reduced fees under 35 U.S.C. §§ 41(a) and (b), in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons.

DEFINITIONS: For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that the rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention of the above-identified application or patent. If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 35 C.F.R. § 1.9(d) or by any concern which would not qualify as a small business concern under 37 C.F.R. § 1.9(d) or a nonprofit organization under 35 C.F.R. § 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities (37 C.F.R. § 1.27).

NAME: ERUME BIOTECH CO., LTD.

ADDRESS: 4th Fl. Erume Building, 807-3, Bangbae-dong, Seocho-ku, Seoul
137-060, Republic of Korea

☐ INDIVIDUAL ☒ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 C.F.R. § 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statement and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: Jae Hyuk, NOH

TITLE OF PERSON (IF OTHER THAN OWNER): President

ADDRESS OF PERSON SIGNING: 4th Fl. Erume building, 807-3, Bangbae-dong, Seocho-ku,
Seoul, 137-060, Republic of Korea

SIGNATURE: Jae Hyuk Noh

DATE: Oct. 24, 2000

rpoB gene fragments and a method for the diagnosis and identification of *Mycobacterium tuberculosis* and non-tuberculosis *Mycobacterium* strains

5

FIELD OF THE INVENTION

The present invention is related to rpoB gene fragments and a method for the diagnosis and identification of *Mycobacterium tuberculosis* and non-tuberculosis
10 *Mycobacterium* strains using rpoB gene fragments.

BACKGROUND OF THE INVENTION

Since the early 1980s, there has been an increase in disease caused by
15 organisms called nontuberculous mycobacteria (NTM), which is the generic name for mycobacteria other than *M. tuberculosis* and *M. leprae* (MOTT). They affect both immune-competent and immune-compromised persons, and patients with the human immunodeficiency virus (HIV) are known to be especially vulnerable. The most frequent NTMs involved in disease cases are known to be *M. avium*, *M. intracellulare*,
20 *M. scrofulaceum*, *M. kansasii*, *M. fortuitum* complex, *M. chelonae*, *M. abscessus*, *M. szulgai*, *M. malmoense*, *M. marinum*, *M. ulcerans*, and *M. africanum*, *M. bovis* (28). Clinical diagnosis and treatment of nontuberculous mycobacterial infections are an increasingly frequent challenge to clinicians.

Currently, clinical diagnosis of mycobacteria to the species level is primarily
25 based on cultural and biochemical tests. These conventional tests take several weeks, and the tests sometimes fail precise identification. The procedures for these tests are complex, laborious, and are usually impeded by the slow growth of mycobacteria in clinical laboratories. Additional methods, such as high-performance liquid

chromatography, gas-liquid chromatography, thin-layer chromatography (5, 21, 36), and DNA sequencing analysis (3, 4, 15, 16, 17, 19, 26, 31, 32) can differentiate mycobacteria to the species level, but these are labor-intensive and difficult to perform for routine use in many clinical laboratories.

5 In contrast to the above-mentioned techniques, recent molecular techniques employing PCR-amplified products offers an easy, rapid, and inexpensive way to identify several mycobacterial species in a single experiment. PCR-restriction fragment length polymorphism analysis (PRA) has been developed to target mycobacterial genes, which are present in all mycobacteria such as *hsp65* (7, 11, 25, 29, 30, 34, 35), 16S
10 rRNA (2, 14, 37), and *dnaJ* (33). However, these techniques are still cumbersome since they require several enzyme digestions for species identification, and the results are not easy to interpret for species identification due to the limited size variation of DNA fragments after digestion.

On the other hand, probe-hybridization technique which employs DNA of the
15 clinical specimen and oligo-probe hybridization (8, 9, 10, 18, 20, 23) is a useful tool for direct and rapid identification of NTM species. However, commercial kits currently available in the market are very expensive, limited only to 5 mycobacterial species, and the identification of a single species requires an independent experiment.

20 SUMMARY OF THE INVENTION

The present invention provides DNA fragments including sequence SEQ. ID. NO. 1 to 4 and 6 to 24.

The present invention provides a method of identification of Mycobacterium
25 strain comprising the step of 1) digesting a DNA fragment which has one of the sequence Seq. ID. NO 1 to 4 to 24 with restriction enzyme to obtain DNA fragment

length polymorphism pattern ; 2) isolating DNA fragment from microorganism to identify; 3) amplifying said DNA fragment; 4) digesting said amplified DNA fragment with the same restriction enzyme in step 1) ; 5) obtaining DNA fragment length polymorphism pattern from DNA fragment in step 4) ; and 6) comparing DNA fragment length polymorphism pattern from step 1) with DNA fragment length polymorphism pattern from step 5).

Preferably, said restriction enzymes are enzyme HaeIII, MspI, Sau3A1 or BstEII.

Preferably, the DNA fragment length polymorphism pattern from steps 1) and 5) is obtained by electrophoresis.

And the Mycobacteria strain to be identified by this method are preferably *M. tuberculosis*, *M. avium*, *M. abscessus*, *M. flavescence*, *M. africanum*, *M. bovis*, *M. chelonae*, *M. celatum*, *M. fortuitum*, *M. gordonae*, *M. gastri*, *M. haemophilum*, *M. intraecellulare*, *M. kansasii*, *M. malmoeense*, *M. marinum*, *M. szulgai*, *M. terrae*, *M. scrofulaceum*, *M. ulcerans* or *M. xenopii*.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. (A). A diagram showing amplified region of the *rpoB* for PRA in this study. The primers PRO5' and RPO3' generates 360-bp PCR product, which locates upstream of *rif^r* region associated with resistance of *M. tuberculosis* to rifampin.

(B). An agarose gel (2%) with 360-bp PCR products using PRO5' and RPO3'. Lane M. DNA size marker (100-bp ladder), lane 1: negative control (no DNA sample), lanes 2-11: PCR products with reference strains of mycobacteria.

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Fig. 2. An example of PRA results with reference strains of mycobacteria using a set of primers (RPO5' and RPO3'). Amplified DNA was digested using both (A) *Msp* I and (B) *Hae* III restriction enzymes, and run on a 4% Metaphore agarose gel. Lane M:

DNA size marker (50-bp ladder), lane 1: *M. gordonae* type IV, lane 2: *M. szulgai*, lane 3: *M. kansasii* type I, lane 4: *M. gallinarum*, lane 5: *M. avium*, lane 6: *M. scrofulaceum*, lane 7: *M. asiaticum*, lane 8: *M. chelonae*, lane 9: *M. moriokaese*, lane 10: *M. phlei*, lane 11: *M. pulveris*, lane 12: *M. fortuitum* type I, lane 13: *M. austroafricanum*, lane 14: *M. smegmatis*, lane 15: *M. marinum*.

Fig. 3. PRA results with clinical isolates that have been identified by conventional methods, including microbiological and biochemical tests. PCR products were digested with *Msp* I enzyme and electrophoresed on 4% Metaphore agarose gel. Strains were clinical isolates of (A) *M. kansasii*, (B) *M. tuberculosis*, and (C) *M. chelonae* complex that include *M. chelonae sub. chelonae* and *M. chelonae sub abscessus*.

Fig. 4. An algorithm was constructed based on the results of PRA with 40 mycobacterial reference strains and 3 other related bacterial strains. The PRA results of 10 other mycobacterial reference strains are not listed in this figure to make the algorithm concise.

Fig. 5. An example of the application of *rpoB*-based PRA for the species identification of mycobacterial clinical isolates in clinical laboratory. Clinical isolates were amplified using primers, RPO5' and RPO3', digested with *Msp* I, and run on a 4% Metaphore agarose gel. A DNA size marker (lane M: 50-bp ladder) and the PRA result of *M. bovis* was used as an internal size marker (lane 16) for each test. Using the algorithm in Fig. 3, these clinical isolates were determined to be *M. intracellulare* (lanes 1-6, 8, 9, 11-15), *M. gordonae* type II (lane 7), and *M. abscessus* (lane 10).

Fig. 6. Sequence alignment of the *rpoB* region amplified using a set of primers RPO5' and RPO3' from 35 different mycobacterial species. Sequences were aligned using multi-align program(6). Dashed lines represent nucleotide gaps.

5 Fig. 7. Examples of PCR-dot blot hybridization experiments. A total of 48 PCR products generated by using primers, RPO5, and RPO3', and DNAs from 48 mycobacterial species were blotted on the membrane, and an oligonucleotide probe which is specific to a certain mycobacterial species was hybridized at conditions described in the Materials and Methods section. Blotted DNAs on the membrane were

10 as following; 1: *M. tuberculosis*, 2: *M. scrofulaceum* 3: *M. szulgai*, 4: *M. gastri*, 5: *M. kansasii* type I, 6: *M. kansasii* type II, 7: *M. kansasii* type III, 8: *M. kansasii* type IV, 9: *M. kansasii* type V, 10: *M. terrae*, 11: *M. avium*, 12: *M. intracellulae*, 13: *M. africanum*, 14: *M. celatum* type I, 15: *M. celatum* type II, 16: *M. haemophilum*, 17: *M. malmoeense*, 18: *M. bovis*, 19: *M. chelonae*, 20: *M. abscessus*, 21: *M. ulcerans*, 22: *M. marinum*, 23: *M. genevansae*, 24: *M. simiane*, 25: *M. flavescens*, 26: *M. fortuitum* type I, 27: *M. fortuitum* type II, 28: *M. peregrinum*, 29: *M. triviale*, 30: *M. phlei*, 31: *M. parafortuitum*, 32: *M. vaccae*, 33: *M. aurum*, 34: *M. neoaurum*, 35: *M. fallax*, 36: *M. xenopi*, 37: *M. aichiense*, 38: *M. mucogenicum*, 39: *M. nonchromogenicum*, 40: *M. senegalense*, 41: *M. smegmatis*, 42: *M. thermoresistable*, 43: *M. intermedium*, 44: *M. gordonae* type I, 45: *M. gordonae* type II, 46: *M. gordonae* type III, 47: *M. gordonae* type IV, 48: *M. bovis*, BCG

DETAILED DESCRIPTION OF THE INVENTION

25 Mycobacterial identification to the species level is not only of academic

interest but also is important because it provides a great deal of useful information on the epidemiology and pathogenesis of the organism, suggesting potential intervention strategies including successful treatment of patients on the clinical base. It is therefore important to develop methods that are rapid and simple, but yet precise and cost-effective to be used in a wide variety of clinical laboratories around the world. Currently available methods for differentiation of mycobacteria to the species level are time-consuming evaluations using phenotypic and biochemical tests or laborious procedures using expensive equipment.

As compared to other molecular methods, the PRA method certainly fits these requirements better. It is rapid and precise since it employs PCR, and simple and cost-effective since it does not require any expensive equipment or laborious processes and can differentiate numerous species of mycobacteria within a single experiment. Owing to these advantages, several PRA methods based on different genes of mycobacteria have been developed (2, 7, 11, 14, 25, 29, 30, 33, 34, 35, 37). However, most of those methods require use of more than two enzymes to differentiate mycobacteria at the species level, and require computer-assisted software program to differentiate restriction fragments since the profiles of certain mycobacterial species were not distinctive enough for bare-eye interpretation.

The new PRA method developed through this invention has more advantages than the previous ones. As presented in Fig. 1, it is apparent that most of the species harbor unique PRA profiles. Unlike other PRA profiles, which may need computer-assisted analysis and interpretation of the gels, we do not face problems in resolving all the patterns obtained during the experiments. Furthermore, problems including gel-to-gel variations or confusion with the size of the restriction fragments were limited with the use of 50-bp size marker and PRA profile of *M. bovis* as an internal size marker.

On the other hand, the four members of the *M. tuberculosis* complex that are difficult to separate by using other methods such as sequence analysis or HPLC of mycolic acids were also undistinguishable by PRA method, confirming that they do belong to a genetically similar group. However, unlike other methods, this new PRA method can further differentiate *M. africanum* from other *M. tuberculosis* complex by *Sau* 3AI digestion. Therefore, in case the clinical isolate shows the *M. tuberculosis* complex profiles, PCR products can be further processed to differentiate *M. africanum* from other *M. tuberculosis* complex by *Sau* 3AI digestion. In addition, *M. tuberculosis* and *M. bovis* can be differentiated by PCR amplification using *esat-6* gene derived PCR primers, which is known to be present only in the genome of *M. tuberculosis*.

Currently in our laboratory, a substantial number of mycobacterial clinical isolates have now been identified by our new PRA method in parallel with other reference methods, including conventional tests and molecular biological methods such as PRA based on *hsp65* gene and sequence analysis based on the *rpoB* gene. As a conclusion of this experiment, it is certain that this new PRA is a rapid, cost-effective, and efficient method for the identification of mycobacteria in a clinical microbiology laboratory. The whole procedure can be done in 2 days when culture is used. PRA has been successful when using a loopful of culture taken from solid media or using 100 µl taken from liquid culture such as MGIT for mycobacterial species identification. Both of systems work well even with genomic DNA simply boiled for 5 min.

In addition to the PRA, PCR-dot blot and PCR-reverse dot blot hybridization method employing oligonucleotide probes that are highly specific to each mycobacterial species were also shown to be valuable techniques for simple and rapid identification of mycobacterial species. The oligonucleotides developed in this study were highly species-specific, thus indicating a usefulness of these probes in development of

mycobacterial identification system which can be useful in clinical settings.

To develop new molecular techniques that are easier and more precise for mycobacterial species identification than currently available ones, we chose the *rpoB* gene that encodes β subunit of RNA polymerase. The information-rich nature of the *rpoB* gene has been recently employed in differentiation of mycobacteria by DNA hybridization array (10) or by DNA sequence analysis (16). However, the *rpoB* region used in these previous studies has limited sequence variation that can be used for species identification of mycobacteria. In the present study, we extended the genetic knowledge of the *rpoB* gene to the highly polymorphic region that is suitable for developing mycobacterial species identification system using molecular biological techniques such as PRA and PCR-DNA hybridization. We also chose this region of the *rpoB* gene to be flanked by highly conserved sequences, thus can be suitable for PCR amplification of the *rpoB* region of all mycobacterial species using the same set of PCR primers.

In this study, 50 reference strains representing 44 different mycobacterial species and 6 subspecies were used to amplify the 360-bp region of the *rpoB* gene. The PCR products were then subjected to restriction fragment length polymorphism analysis (RFLP) to determine the efficacy of this region of the *rpoB* gene for mycobacterial species identification using PRA method. Subsequently, on the basis of PRA profiles generated with reference strains, an algorithm was generated, and a total of 260 clinical isolates were evaluated using new PRA method. In brief, the results clearly showed that this novel PRA method based on the *rpoB* gene generates clear and distinctive results for easy, rapid, and precise identification of mycobacterial species that can be employed in clinical laboratories for prompt and accurate diagnosis.

Subsequently, PCR amplified regions of the *rpoB* gene derived from 30

mycobacterial species that are known to have clinical importance were sequenced. In brief, results of sequence analysis showed that in the region of *rpoB* we amplified, highly polymorphic and species-specific regions exist, and thus indicated the usefulness of these regions for developing a new PCR-dot blot hybridization technique. On the basis of these sequence information, species-specific oligo-probes were designed and used to establish mycobacterial species identification system using DNA hybridization techniques such as PCR-dot blot and PCR-reverse blot hybridization method.

The restriction analysis of a 360-bp fragment within *rpoB* gene after single *Msp* I digestion is highly effective for differentiating most of mycobacteria even at the species level. Only several species require additional enzyme digestion such as *Hae* III, *Sau* 3AI, *Hinc* II, etc. For some species, such as *M. gordonae*, *M. kansasii*, *M. fortuitum*, and *M. celatum*, the discrimination was even obtained at the subtype level. For *M. kansasii*, this subdivision was clearly linked to genetic divergence observed previously by other investigators (1, 24, 27). It is therefore possible that using this PRA method, the discrimination at a subgroup level for other species could be similarly linked to bacteriological and clinical specificities.

Therefore, this invention provide a *rpoB* gene fragment(SEQ. ID. NO. 1 to 4 and 6 to 24) which has conserved sequence and polymorphic sequence between mycobacterial species.

Also this invention provide a method for diagnosis and identification of Mycobacterium tuberculosis and Non-tuberculosis Mycobacterium strain comprising the step of

- 1) digesting a DNA fragment which has one of the sequence Seq. ID. NO 1 to 24 with restriction enzyme to obtain DNA fragment length polymorphism pattern ;

- 2) isolating DNA fragment from microorganism to identify;
- 3) amplifying said DNA fragment using primer (SEQ. ID. NO. 25 and 26);
- 4) digesting said amplified DNA fragment with the same restriction enzyme in step 1) ;
- 5) obtaining DNA fragment length polymorphism pattern from DNA fragment in step 4) ; and
- 6) comparing DNA fragment length polymorphism pattern from step 1) with DNA fragment length polymorphism pattern from step 5).

Preferably, said restriction enzymes are enzyme HaeIII, MspI, Sau3A1 or BstEII.

Preferably, the DNA fragment length polymorphism pattern from steps 1) and 5) is obtained by electrophoresis.

And the Mycobacteria strain to be identified by this method are listed in Table 1.

Though the present invention has been described with regard to its preferred embodiments, one skilled in the art will appreciate from a reading of this disclosure that various changes in form and detail can be made without departing from the scope and spirit of the invention.

EXAMPLES

MATERIALS and METHODS

Mycobacterial samples. A total of 50 mycobacterial reference strains representing 44 mycobacterial species and 3 related species which belong to 2 different genera (Table 1) were used to develop the new PRA method in this study. Among them, 40 mycobacterial strains and 3 related species were obtained from the Korean Institute of

Tuberculosis (KIT) and the Korean National Tuberculosis Association (KNTA) in Seoul, Korea. Four species were obtained from the Korean Collection for Type Cultures (KCTC) at the Korean Research Institute of Bioscience & Biotechnology (KRIBB) and *M. abscessus*, which was recently separated from *M. chelonae* as an independent new species, was obtained from Department of Clinical Pathology at Yonsei University Medical College (YUMC). Five subtypes of *M. kansasii* were generously given by Dr. V. Vincent in the Laboratoire de Référence des Mycobactéries, Institut Pasteur in France.

Clinical isolates subjected for PRA to evaluate the new method were obtained from KIT. All clinical isolates used in this study were identified on the basis of conventional tests that include microbiological characteristics and biochemical tests. For some cases, strains were subjected for conventional PRA method based on *hsp65* gene (7, 35) to help precise identification of clinical isolates.

Table 1. Bacterial strains used in this study

	Species	Strain	Source
1	<i>M. abscessus</i>	Pettenkofer Inst.	YUMC
2	<i>M. africanum</i>	ATCC 25420	KIT
3	<i>M. arcinogenes</i>	ATCC 35753	KIT
4	<i>M. asiaticum</i>	ATCC 25276	KIT
5	<i>M. aurum</i>	ATCC 23366	KIT
6	<i>M. austroafricanum</i>	ATCC 33464	KRIBB
7	<i>M. avium</i>	ATCC 25291	KIT
8	<i>M. bovis</i>	ATCC 19210	KIT
9	<i>M. bovis</i> BCG	French Strain 1173P2	KIT
10	<i>M. celatum</i> type I/II	ATCC 51130/ATCC 51131	KIT
11	<i>M. chelonae</i>	ATCC 35749	KIT
12	<i>M. chitae</i>	ATCC 19627	KIT
13	<i>M. fallax</i>	ATCC 35219	KIT
14	<i>M. fortuitum</i> type I/II	ATCC 6841/ATCC 49404	KIT
15	<i>M. gallinarum</i>	ATCC 19710	KRIBB
16	<i>M. gastri</i>	ATCC 15754	KIT

17	<i>M. genavense</i>	ATCC 51233	KIT
18	<i>M. gilvum</i>	ATCC 43909	KIT
19	<i>M. gordonae</i>	ATCC 14470	KIT
20	<i>M. haemophilum</i>	ATCC 29548	KIT
21	<i>M. intracellulare</i>	ATCC 13950	KIT
22	<i>M. interjectum</i>	ATCC 51457	KIT
23	<i>M. intermedium</i>	ATCC 51848	KIT
24	<i>M. kansasii</i> type I-V		Pasteur Inst.
25	<i>M. malmoense</i>	ATCC 29571	KIT
26	<i>M. marinum</i>	ATCC 927	KIT
27	<i>M. moriokaense</i>	ATCC 43059	KRIBB
28	<i>M. mucogenicum</i>	ATCC 49650	KIT
29	<i>M. neoaurum</i>	ATCC 25795	KIT
30	<i>M. nonchromogenicum</i>	ATCC 19530	KIT
31	<i>M. parafortuitum</i>	ATCC 19686	KIT
32	<i>M. peregrinum</i>	ATCC 14467	KIT
33	<i>M. phlei</i>	ATCC 11758	KIT
34	<i>M. pulveris</i>	ATCC 35154	KRIBB
35	<i>M. scrofulaceum</i>	ATCC 19981	KIT
36	<i>M. smegmatis</i>	ATCC 19420	KIT
37	<i>M. szulgai</i>	ATCC 35799	KIT
38	<i>M. terrae</i>	ATCC 15755	KIT
39	<i>M. thermoresistibile</i>	ATCC 19527	KIT
40	<i>M. triviale</i>	ATCC 23292	KIT
41	<i>M. tuberculosis</i> H37Rv	ATCC 27294	KIT
42	<i>M. ulcerans</i>	ATCC 19423	KIT
43	<i>M. vaccae</i>	ATCC 15483	KIT
44	<i>M. xenopi</i>	ATCC 19250	KIT
45	<i>N. brasiliensis</i>	ATCC 19296	KIT
46	<i>N. nova</i>	ATCC 21197	KIT
47	<i>R. equi</i>	ATCC 10146	KIT

DNA preparation. In order to prepare a DNA sample for PCR amplification, a loopful of bacterial colony was taken from the Löwenstein-Jensen medium and resuspended in 400 µl of distilled water in a screw-cap microcentrifuge tube. The sample was then boiled for 5 min, centrifuged for 5 min to settle down cell debris, and about 10 µl of supernatant containing

PCR amplification. The primer set used to amplify the region of the *rpoB* were 5'-TCAAGGAGAAGCGCTACGA-3'(RPO5') and 5'-GGATGTTGATCAGGGTCTGC-3' (RPO3') resulting in about 360-bp PCR product (base number 902 to 1261

and codon number 302 to 420 based on the sequence numbers for the *rpoB* gene of *M. tuberculosis* [GenBank accession No. p47766]. The primer sequences were selected from the region of the *rpoB* genes that have been previously identified from *M. tuberculosis*, *M. leprae*, and *M. smegmatis* (12, 13, 22). The primers were made to
5 amplify the region between the variable region and conserved region based on the genetic information for the *rpoB* gene of *Escherichia coli*. As a result, PCR products included 171-bp of variable region and 189-bp of conserved region. Variable region was amplified in this experiment based on an assumption that the polymorphic nature of this region might help the clear distinction of each mycobacterial species using molecular
10 biological techniques such as PRA and PCR-DNA hybridization. On the other hand, the region of the *rpoB* gene was also chosen to be flanked by highly conserved sequences, thus can be suitable for PCR amplification of the *rpoB* region of all mycobacterial species using the same set of PCR primers.

PCR was carried out in a final volume of 50 μ l with 10 μ l of DNA supernatant
15 containing approximately 10 ng of genomic DNA, 10 pmole of each primers, 2 mM $MgCl_2$, 200 μ M of deoxynucleotide triphosphates, and 1 unit of DyNAzymeTMII DNA polymerase (FINNZYMES, Espoo, Finland). DNA samples were first denatured completely by incubation at 94°C for 5 min before amplification cycle, then amplified using a cycle that includes (1) denaturation at 94°C for 1 min, (2) primer annealing at
20 58°C for 1 min, and (3) elongation at 72°C for 1 min for 35 times using a Thermocycler (model 9600, Perkin Elmer). After the last amplification cycle, the samples were incubated further at 72°C for 7 min for complete elongation of the final PCR products. Positive and negative controls were always included in each PCR reaction. The positive control was the PCR mix with DNA of reference strain, *M. bovis*, and the negative
25 control was the PCR mix without any DNA. After the PCR, the amplification results

were visualized using 1.5% agarose gel electrophoresis and ethidium bromide staining.

Restriction fragment length polymorphism analysis. After successful amplification, the 360-bp long PCR products were subjected to restriction enzyme digestion. Most of the time, 16 µl of PCR products (approximately 1 to 1.5 µg of DNA) were digested in a 20 µl of reaction volume using 5 units of *Msp* I (Boehringer Mannheim Biochemicals, Mannheim, Germany) and 2 µl of 10X reaction buffer supplied by manufacturer. Similarly, 16 µl of PCR product was digested in a 20 µl of reaction volume containing 5 units of *Hae* III enzyme (Takara Shuzo Co., LTD., Shiga, Japan) with the corresponding enzyme buffer. If necessary, additional enzyme digestions were carried out in a similar reaction condition. After 2 hours of incubation at 37° C, 4 µl of gel loading buffer (0.25% bromophenol blue, 40% sucrose in water) was added, and the samples were loaded into a 4% metaphore agarose gel (FMC BioProducts, Rockland, Maine). Then, enzyme digested fragments were visualized by ethidium bromide staining and UV- light.

For the interpretation of the PRA profiles generated by each species, 50-bp ladder DNA size marker (Boehringer Mannheim, Germany) and the PRA profile of *M. bovis*, which generates about 175-bp, 80-bp, 60-bp, 40-bp restriction fragments, were used as an internal size marker. Using these size markers, the sizes of the restricted fragments of each species were determined, and an algorithm was made based on this information.

Cloning and sequence analysis. For sequence analysis, PCR products were purified by using a GeneClean kit (BIO101, Vista, Calif. USA) from an agarose gel and cloned into TOPO-TA cloning vector (Invitrogen Co., Carlsbad, CA) by the method

recommended by the manufacturer. DNA sequencing was done by the dideoxy nucleotide-chain termination method (21) using ARL automatic sequencer (Pharmacia Biotech, Uppsala, Sweden). For each clone, M13 reverse primer and T7 promoter primer were used separately to read sequences from both directions. Sequences were aligned using a multiple sequence alignment program (6)

Oligonucleotide probes used in PCR-DNA hybridization assay Oligonucleotide probes for detecting specific mycobacterial species were designed to be 15-17 nucleotide long, and to contain 10-11 G+C content (Table 2). However, the oligonucleotide probe for all the mycobacterial species (named as "Pan-TB" probe) was designed to be 20 nucleotide long. These specific oligonucleotide length and G+C content were selected, so that the hybridization conditions for each oligonucleotide to each mycobacterial DNA to be about the same.

Table 2. Oligonucleotide probes designed in this study to develop PCR-probe hybridization assay for Mycobacterial species identification

Name of oligonucleotides	Sequences of oligonucleotides	target mycobacteria
PAN-MYC	GACGTCGTCGCCACCATCGA	All mycobacterial species
TB	CATGTCGGCGAGCCC	<i>M. tuberculosis</i> complex
AVIUM	CGGTGAGCCGATCACCA	<i>M. avium</i>
INTRA	CCTGCACGCGGGCGA	<i>M. intracellulae</i>
GORDONAE	GTCGGCGATCCGATCA	<i>M. gordonae</i>
SZULGAI	TCTGAACGTCGGCGAG	<i>M. szulgai</i>
KANSASII	GGCCACGATGACCGTG	<i>M. kansasii</i>
GASTRI	TCTGAACGTCGGCGAG	<i>M. gastri</i>
FORTUITUM	CCTGAACGCCGGCCAG	<i>M. fortuitum</i>

FORTUITUM-COM	GTTCCGGTCGAGGTGG	<i>M. fortuitum complex</i>
SCROFULACEUM	CGTACGGATGGCCAGC	<i>M. scrofulaceum</i>
CHELONAE	TGGTGACTGCCACCACG	<i>M. chelonae</i>
ABSCCESSUS	AGGTGACCACCACCACC	<i>M. abscesus</i>
TERRAE	GCTCAGGACGGTCAGT	<i>M. terrae</i>
ULCERANS/MARINUM	GGCCAGCCCATCACC	<i>M. ulcerans / M. marinum</i>
GENAVANSE/SIMIAE	CCAGCCGACGATGACG	<i>M. genavanse / M. simiae</i>

PCR-dot blot hybridization. To prepare the DNA dot blot, pre-cut (10x10cm²) membrane (Hybond-N⁺; Ammersham) was immersed into the denaturing solution (0.4N NaOH, 25 mM EDTA; pH 8.0) for 1 min. After dripping excess amount of denaturing solution, the membrane was placed on the 3MM paper, and 1-2 µl of PCR product was blotted on the membrane. Then, the membrane was air-dried for 5 min, rinsed with the denaturing solution for another 1 min, placed in-between two sheets of 3MM papers, and baked for 2 hrs at 80°C. Oligonucleotide probes were labeled by using a commercially available kit for 3'-oligolabelling and detection (ECL, Amersham Life Science). Before hybridizing with oligonucleotide probes, membrane was prehybridized at 42°C for 30 min., and subsequently hybridized with 10pmol of labeled oligonucleotide probes at 42°C for 1hr. Then, the membrane was washed twice at room temperature for 20 min, and washed twice again at 52°C for 15min. Subsequent procedures including antibody binding, washing and the signal detection were all carried out by the method recommended by the manufacturer.

PCR-reverse blot hybridization. All oligonucleotide probes to be applied on the membrane were synthesized with 5' terminal amino group, which link the oligonucleotides to the Biodyne C membrane (Pall BioSupport, East Hills, NY) by

forming covalent bond with negatively charged carboxyl group fixed on the membrane. Before blotting the oligonucleotide probes, the Biotodyne C membrane was activated by incubating in 10 ml of freshly prepared 16% (w/v) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC). After rinsed with the water, the membrane was placed on a support cushion in a clean miniblotted system (Immunetics, Inc., Cambridge, MA), and the residual water was removed from the slots. Then, the slots were filled with 150 μ l of the diluted oligonucleotide solutions (approximately 200 pmol to 1 nmol of oligonucleotides in 150 μ l of 500 mM NaHCO₃, pH 8.4). Subsequently, the membrane was incubated for 1 hr at room temperature, and then excess amount of oligonucleotide solution was removed from the slots by aspiration. In order to inactivate the membrane, the membrane was removed from the miniblotted using forceps, incubated in 100 mM NaOH for 10 min in a rolling bottle, and washed in 100 ml 2x SSPE/0.1% SDS for 5 min at 60°C in a plastic container under gentle shaking. Before applying PCR products on the Biotodyne C membrane, the membrane was incubated for 5 min at room temperature in 100 ml 2x SSPE/0.1% SDS.

After placing the membrane on a support cushion into the miniblotted, in such a way that the slots were perpendicular to the line pattern of the applied oligonucleotides, residual fluid was removed from the slots by aspirations. For hybridization, about 10 μ l of PCR products were diluted in 150 μ l of 2x SSPE/0.1% SDS and heat-denatured for 10 min at 99°C and chilled immediately on ice. The slots were then filled with the diluted PCR products and the membrane was hybridized for 60 min at 42°C. Following hybridization, the membrane was washed in 2x SSPE/0.5% SDS for 10 min at 52°C, and incubated with 10 ml of 1:4000 diluted peroxidase labeled streptavidin conjugate in 2x SSPE/0.5% SDS for 30-60 min at 42°C in a rolling bottle. The membrane was then washed twice in 100 ml of 2x SSPE/0.5% SDS for 10 min at 42°C and rinsed twice with

100 ml of 2x SSPE for 5 min at room temperature. Finally for chemiluminiscent detection of hybridizing DNA, the membrane was incubated for 1-2 min in 20 ml ECL detection liquid and exposed to the x-ray film.

5 RESULTS

Since the genetic information for the *rpoB* genes of some mycobacteria are available, sequences were aligned and searched for regions, which are suitable for PRA. As a result, a set of PCR primer was selected to amplify 360-bp region of the *rpoB*,
10 which contains polymorphic region flanked by conserved regions (Fig. 1. A.).

A total of 50 mycobacterial reference strains and 3 related bacterial strains that belong to the same Actinomycetes class with mycobacteria were used to amplify the 360-bp region of the *rpoB* gene (Table 1). The results showed the amplification of a conserved *rpoB* gene present in all mycobacteria and in some other bacteria such as
15 *Nocardia* and *Rhodococcus* spp. (Fig. 1. B). Subsequently, PCR products were subjected to two sets of restriction enzyme digestion using *Msp* I and *Hae* III individually. These two enzymes were selected on the basis of the sequence information of the *rpoB* gene in *M. tuberculosis*, *M. leprae*, and *M. smegmatis* (12, 13, 22).

20 Based on this information, PCR products were subsequently subjected for RFLP analysis (Fig. 2). In short, the result of this analysis showed that RFLP profiles of PCR products from each mycobacteria species were distinctive each other. *M. kansasii* can be easily differentiated from *M. gastri* which has much in common with non-pigmented variants of *M. kansasii*. In addition, *M. abscessus*, which has been classified as a
25 subgroup of *M. chelonae* and was not easy to be differentiated by conventional

biochemical tests was also differentiated. Furthermore, for some species, such as *M. fortuitum*, *M. cellatum*, *M. goodii* and *M. kansasii* that are known to contain several subtypes, each subtype generated distinctive restriction profiles. Therefore, it clearly indicated that this new PRA method could differentiate mycobacterial species at the species and even at the subspecies level.

Variable RFLP profiles generated with PCR products strongly suggested to us the polymorphic nature of this *rpoB* region amplified by PCR in this study. Then, the next question was whether these variable RFLP profiles were species-specific or also strain-specific. If strains belonging to a certain species also show polymorphic RFLP profiles, it would be too complex to use this region for the mycobacterial species identification. Therefore, clinical isolates that have been identified on the basis of conventional tests were subjected for PRA to determine the species based on an algorithm made from this study by blind tests. The results from this experiment clearly show that there is no variation among different clinical isolates that belong to the same species (Fig. 3).

On the basis of the PRA and sequence analysis results, an algorithm was constructed (Fig. 4). In an algorithm, restriction fragments smaller than 40-bp were omitted in order to reduce the confusion with primer-dimer bands. The fragment sizes are clearly separated from each other, making interpretation of results easier. In brief, the algorithm clearly shows that most mycobacterial species and other related bacterial species can be differentiated at the subspecies level by PRA using *Msp* I and *Hae* III restriction enzymes. In fact, except for several mycobacterial species, most of species can be identified by using a single enzyme, *Msp* I, thus making this new method more useful for mycobacterial species identification than previously developed PRA methods.

For those strains that are not differentiated by two enzyme digestions, the third

enzyme digestion was useful for differentiation. For example, even though the members of *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, and *M. africanum*) were not differentiated by using *Msp* I and *Hae* III, the third enzyme *Sau* 3AI can differentiate *M. africanum* from other members of *M. tuberculosis* complex. In other cases, *Hinc* II can differentiate *M. gordonae* type I from *M. celatum* type I, and etc .

Subsequently, a substantial number of clinical isolates that have been identified on the basis of conventional tests were subjected for PRA (Table 3). In this experiment, a total of 260 clinical isolates were analyzed including *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. fortuitum*, *M. chelonae*, *M. abscessus*, *M. terrae*, *M. gordonae*, *M. szulgai*, etc. For the easy interpretation of the PRA profiles generated by each clinical isolates, a 50-bp ladder size marker was used as a standard size marker, and the PRA profile of *M. bovis* was used as an internal size marker (Fig. 5). Results from the PRA of clinical isolates were evaluated with the help of an algorithm generated on the basis of PRA profiles of reference strains. Most of the PRA results were consistent with conventional test results, while PRA profiles of a few strains were not present in the reference algorithm. Based on the conventional tests and molecular biological sequence analysis, some of these were determined to be "*M. terrae* complex."

Table 3. Clinical isolates of mycobacteria subjected for the species identification using the new PRA.

Species Tested	No. of Clinical Isolates
<i>M. tuberculosis</i>	40
<i>M. avium</i>	40
<i>M. intracellulare</i>	50
<i>M. gordonae</i>	25
<i>M. szulgai</i>	10
<i>M. fortuitum</i>	25

<i>M. chelonae</i>	15
<i>M. abscessus</i>	15
<i>M. kansasii</i>	20
<i>M. terrae</i>	20
Total	260

Next, we sequenced PCR amplified region of the *rpoB* gene derived from 30 mycobacterial species that are known to have clinical importance. Subsequently, the sequences of the amplified regions were analyzed by using a software program (6). The result of the sequence analysis clearly showed that in the region of the *rpoB* we amplified, highly polymorphic regions exist, which are highly species-specific (Fig. 6). This observation suggested to us that this highly polymorphic region of the *rpoB* can be very useful to design mycobacterial species-specific oligonucleotide probes, which can be used for developing a new PCR-dot blot hybridization technique for mycobacterial species identification. Subsequently, based on the sequence information, species-specific oligonucleotide was designed (Table 3), and each oligonucleotide was used as a probe in PCR-dot blot hybridization (Fig. 7). In this experiment, a total of 48 mycobacterial species were blotted on the membrane, and each oligonucleotide was used as a probe to detect specific mycobacterial species. In brief, the results showed that each oligonucleotide probe was shown to be highly specific to each mycobacterial species targeted, indicating the usefulness of oligonucleotides for developing probe-based mycobacterial identification systems such as PCR-dot blot hybridization and PCR-reverse blot hybridization techniques.

Subsequently these probes were used to make a reverse-blot which can be used for the mycobacterial species identification system by using PCR-reverse blot hybridization method. The results showed that the PCR-reverse blot hybridization

method employing each mycobacaterial species-specific oligonucleotide probes are very efficient system for identification of mycobacteria.

All documents cited in the specification and as references below are hereby
5 incorporated in their entirety by reference.

500 400 300 200 100 0

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CLAIMS

1. A DNA fragment which has one of the sequence Seq. ID. NO 1 to 4 or 6 to 24
- 5 2. A method of diagnosis and identification Mycobacterium strain comprising the step of
 - a) digesting a DNA fragment which has one of the sequence Seq. ID. NO 1 to 4 or 6 to 24 with restriction enzyme to obtain DNA fragment length polymorphism pattern ;
 - 10 b) isolating DNA fragment from microorganism to identify;
 - c) amplifying said DNA fragment;
 - d) digesting said amplified DNA fragment with the same restriction enzyme in step a) ;
 - e) obtaining DNA fragment length polymorphism pattern from DNA fragment in
15 step d) ; and
 - f) comparing DNA fragment length polymorphism pattern from step a) with DNA fragment length polymorphism pattern from step e).
3. A method of claim 2, wherein said DNA fragment length polymorphism from step a) and step e) are characterized as obtaining by electrophoresis.
20
4. A method of claim 3, wherein said restriction enzyme is characterized as *HaeIII*, *MspI*, *Sau3A1* or *BstEII*.
- 25 5. A method of claim 2, wherein said Mycobacteria strain is characterized as *M. tuberculosis*, *M. avium*, *M. abscessus*, *M. flavescence*, *M. africanum*, *M. bovis*, *M. chelonae*, *M. celatum*, *M. fortuitum*, *M. gordonae*, *M. gastri*, *M. haemophilum*, *M. intracellulare*, *M. kansasii*, *M. malmoense*, *M. marinum*, *M. szulgai*, *M. terrae*, *M. scrofulaceum*, *M. ulcerans* or *M. xenopii*.

ABSTRACT OF THE DISCLOSURE

The present invention is related to *rpoB* gene fragments and method for the diagnosis and identification of *Mycobacterium tuberculosis* and *non-tubercuolsis*
5 *Mycobacterial* strains using *rpoB* gene and it's fragments.

Figure

Figure 1a

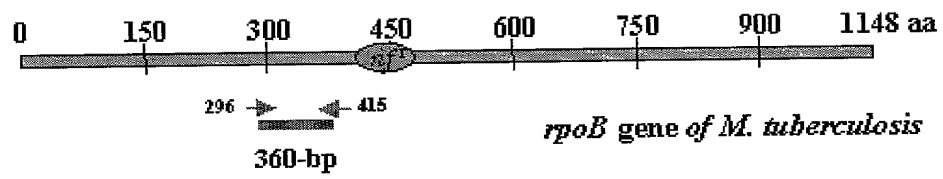


Figure 1b

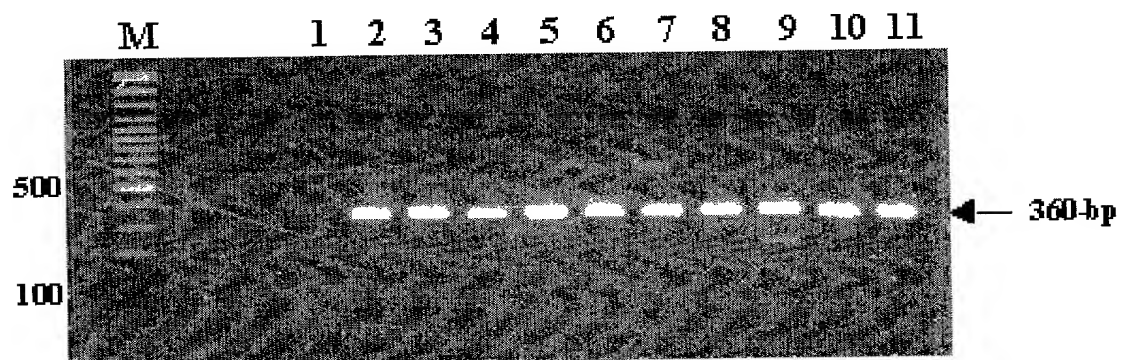


Figure 2a

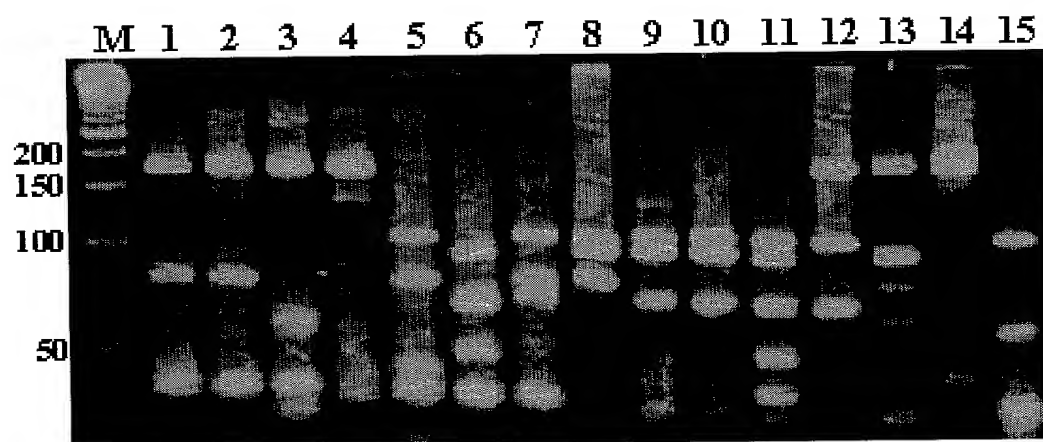


Figure 2b

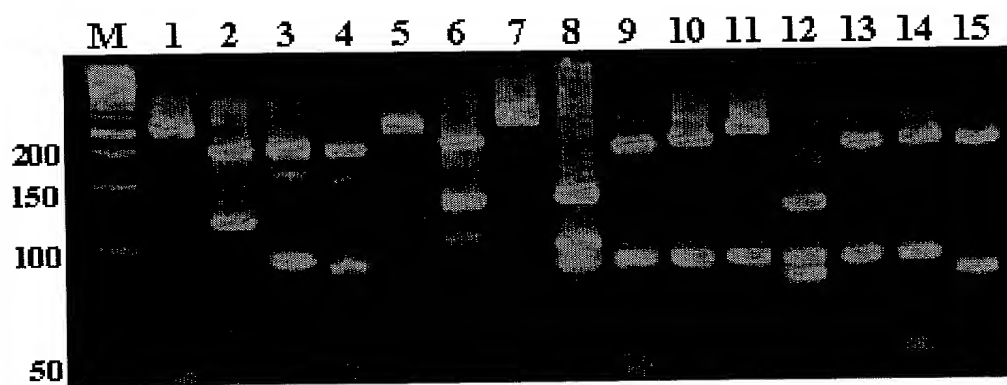


Figure 3a

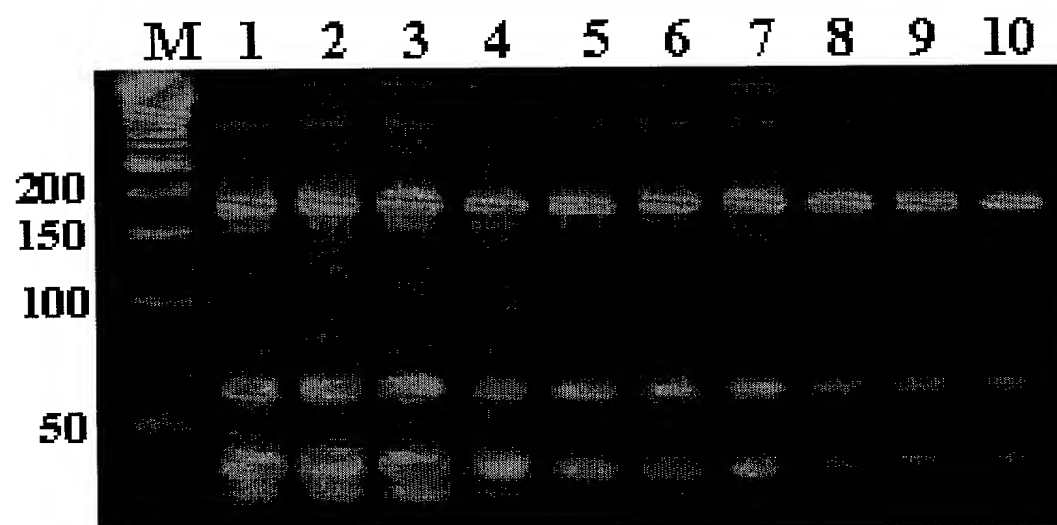


Figure 3b

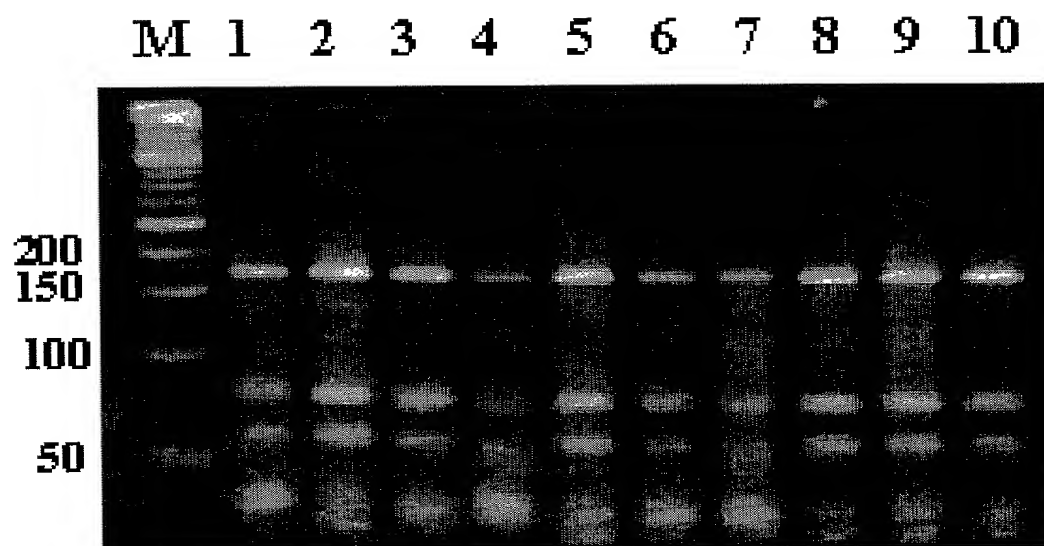


Figure 3c

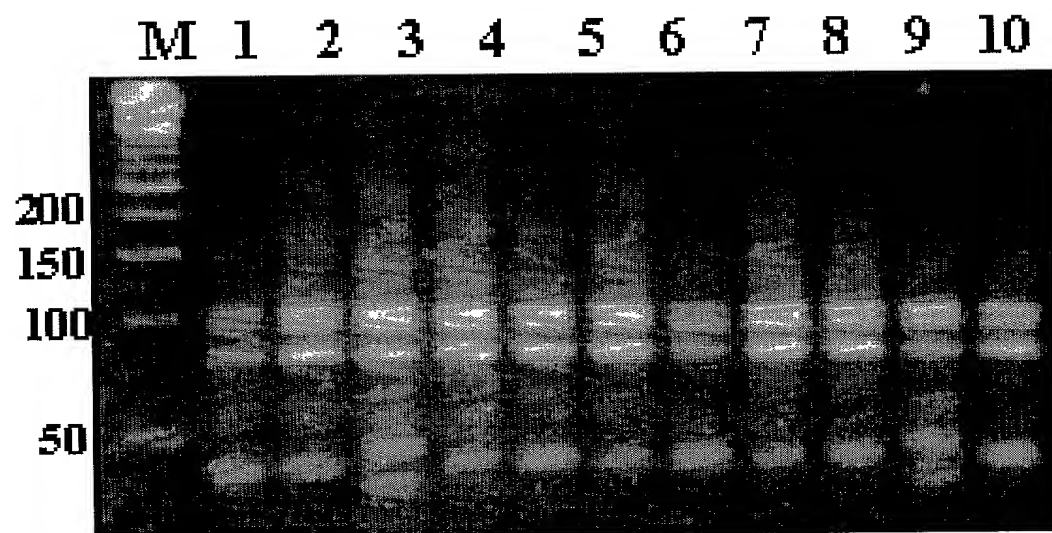


Figure 4

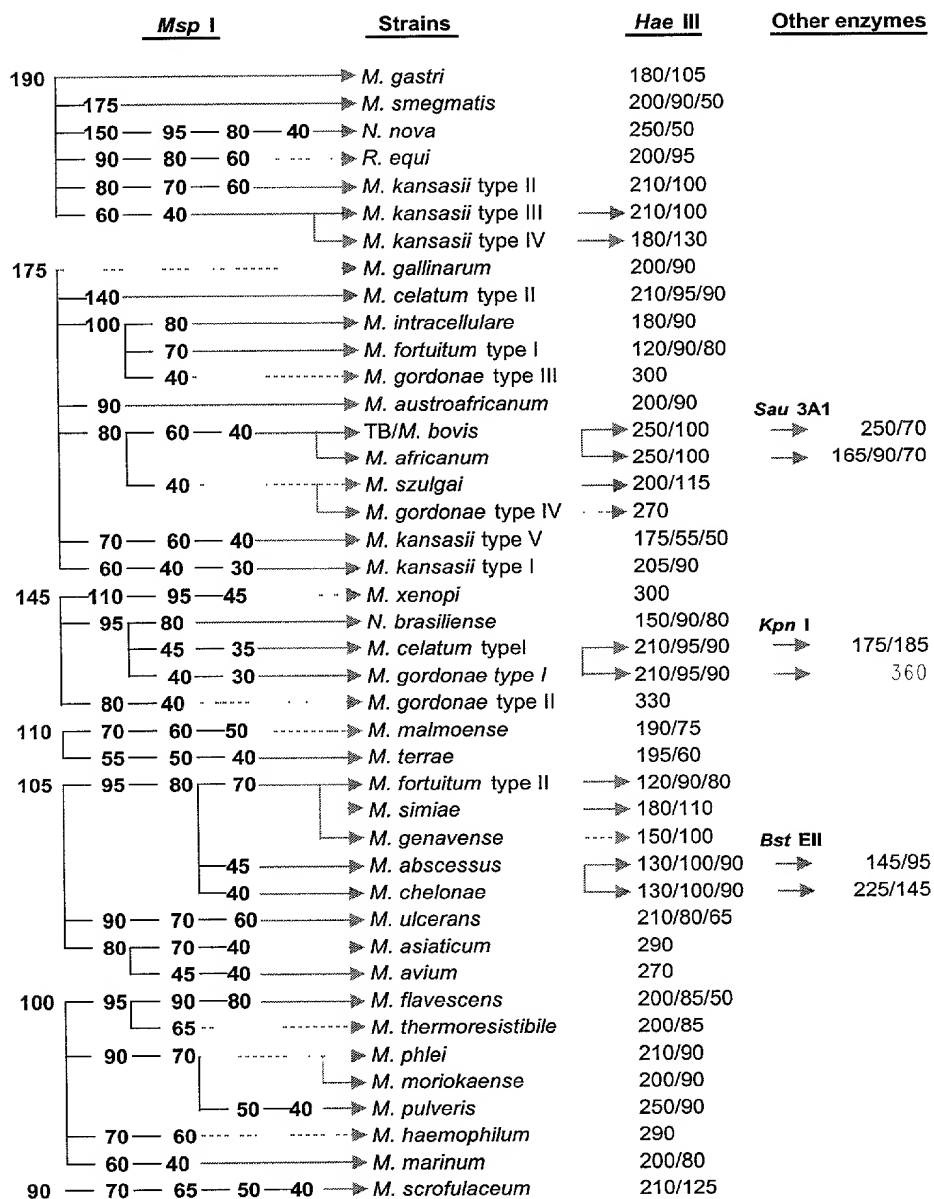


Figure 5

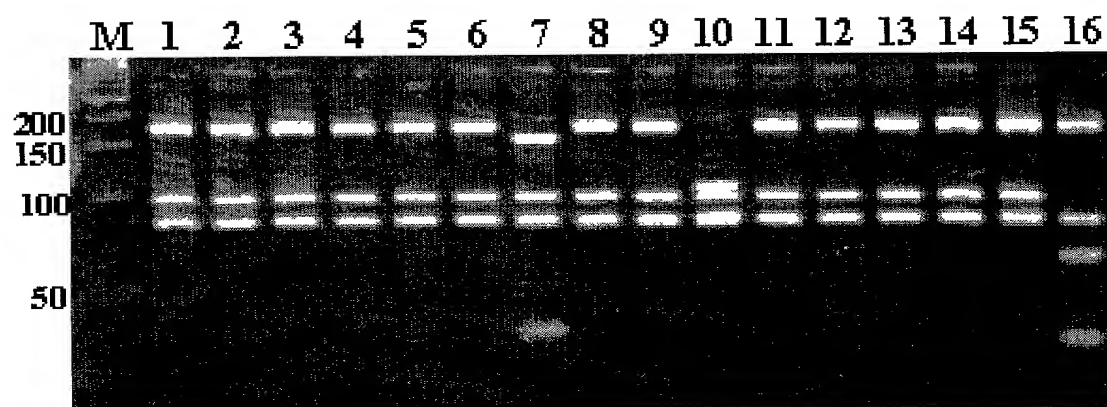


Figure 6a

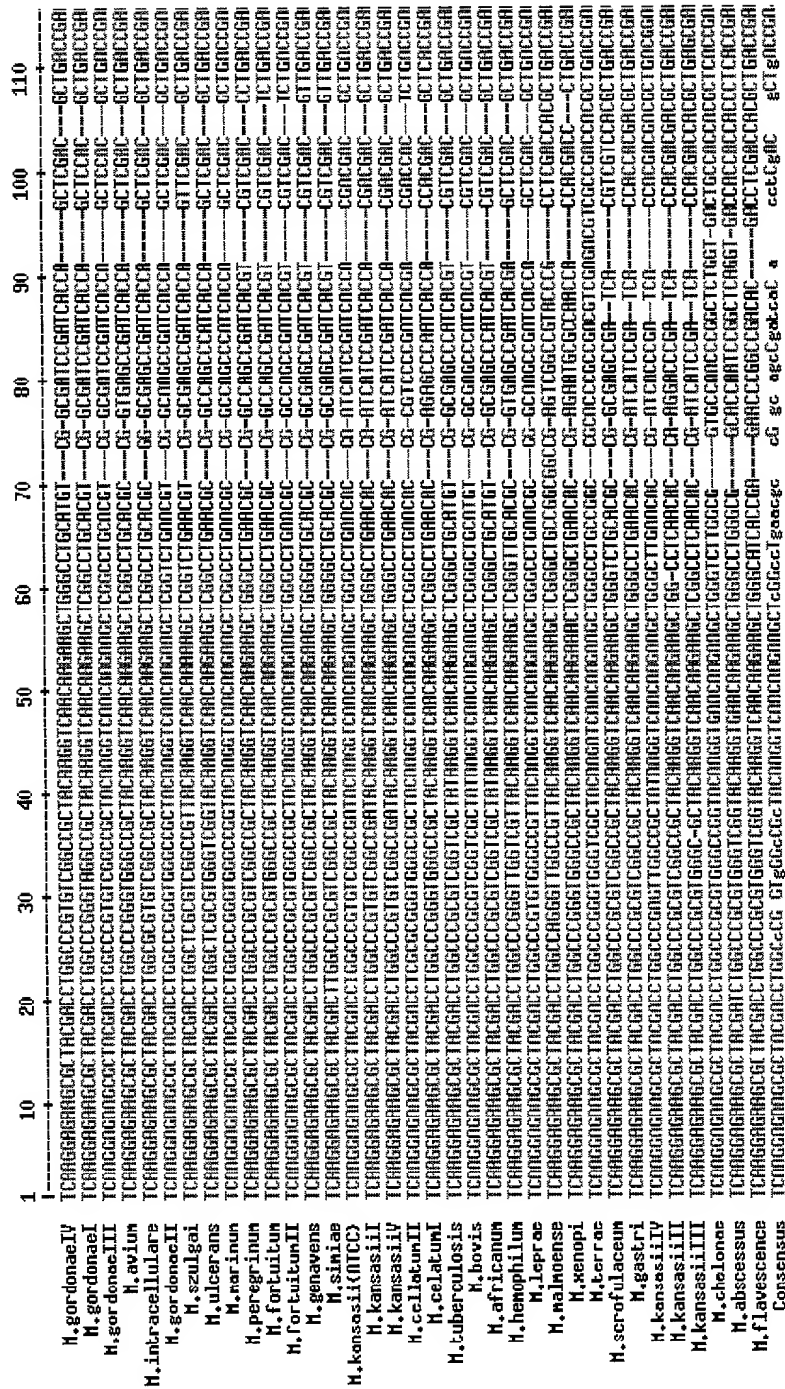


Figure 6b

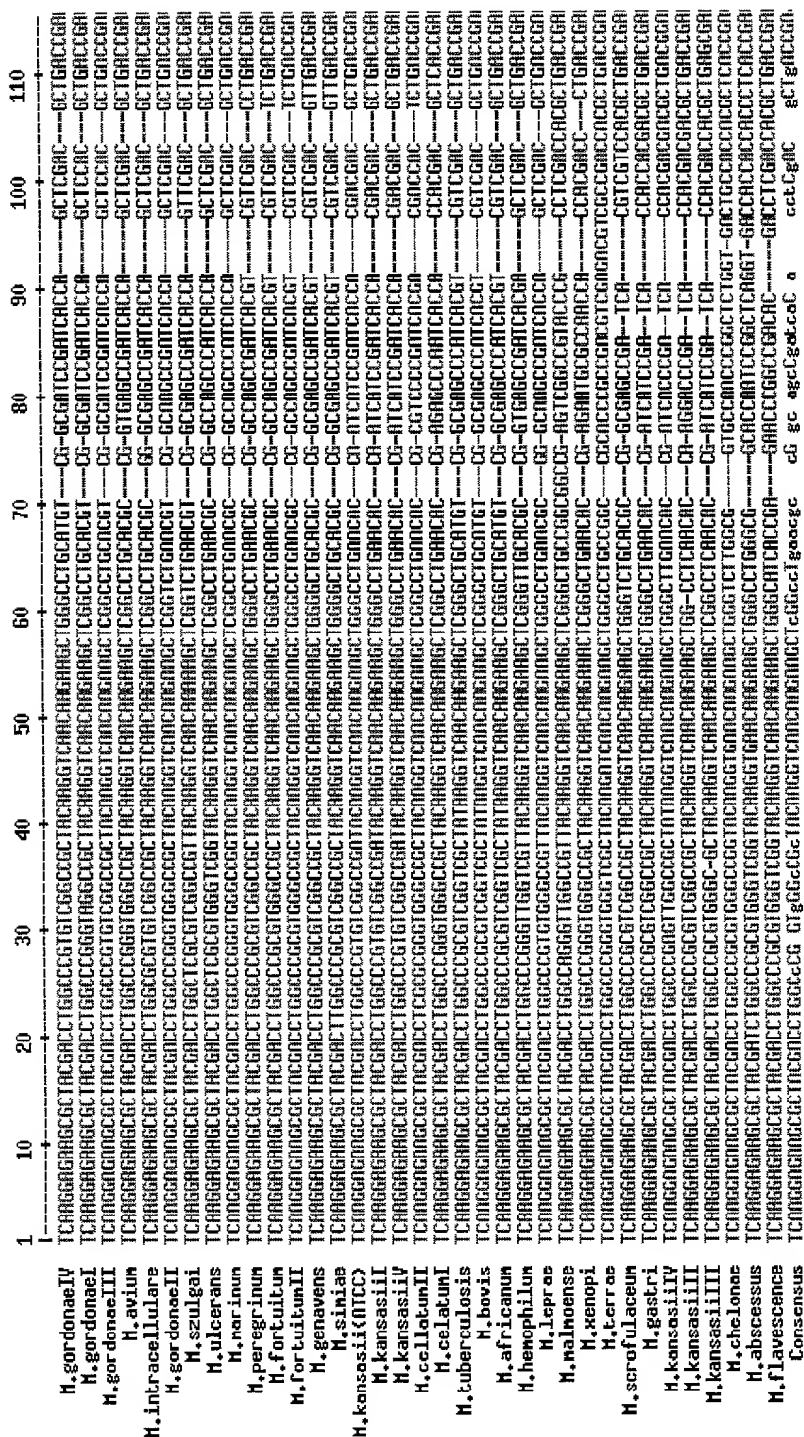


Figure 7a

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31	32	33	34	35	36
37	38	39	40	41	42
43	44	45	46	47	48

Figure 7b

1	2	3	4	5	6
7	8	9	10	11	12
13	14	15	16	17	18
19	20	21	22	23	24
25	26	27	28	29	30
31	32	33	34	35	36
37	38	39	40	41	42
43	44	45	46	47	48

Figure 7c

1	2	3	4	5	6
7	8	9	10	11	12
13	14	15	16	17	18
19	20	21	22	23	24
25	26	27	28	29	30
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37	38	39	40	41	42
43	44	45	46	47	48

COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below, next to my name.

I believe I am the original, first, and sole inventor (if only one name is listed below) or an original, first, and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled rpoB gene fragments and a method for the diagnosis and identification of Mycobacterium tuberculosis and the specification of which:

non-tuberculosis Mycobacterial strains

X is attached hereto.
_____ was filed on _____
as United States Application Number _____
or PCT International Application Number _____
and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment referred to above. I do not know and do not believe that the claimed invention was ever known or used in the United States of America before my invention thereof, or patented or described in any printed publication in any country before my invention thereof or more than one year prior to this application, that the same was not in public use or on sale in the United States of America more than one year prior to this application, and that the invention has not been patented or made the subject of an inventors certificate issued before the date of this application in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns more than twelve months (for a utility patent application) or six months (for a design patent application) prior to this application.

I acknowledge the duty to disclose all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d), of any foreign application(s) for patent or inventors certificate listed below and have also identified below any foreign application for patent or inventors certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

Priority Claimed

<u>1999-46795</u> (Number)	<u>Republic of Korea</u> (Country)	<u>27/10/1999</u> (Day/Month/Year Field)	<u>X</u> Yes	_____ No
_____ (Number)	_____ (Country)	_____ (Day/Month/Year Field)	_____ Yes	_____ No
_____ (Number)	_____ (Country)	_____ (Day/Month/Year Field)	_____ Yes	_____ No

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below

(Application Number)

Filing Date

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Number)	Filing Date	(Status patented, pending, abandoned)
(Application Number)	Filing Date	(Status patented, pending, abandoned)
(Application Number)	Filing Date	(Status patented, pending, abandoned)

I hereby appoint Mark R. Shanks Registration No. 33,781, Toni-Junell Herbert, Registration No. 34,348, Simon D. Roberts (Certificate of Limited Recognition), David W. Woodward, Registration No. 35,020, Keith D. Hutchinson, Registration No. 43,687, Maria L. Maebius, Registration No. 42,967, Mark J. Pino, Registration No. 43,858, Deanna L. Baxam, Registration No. 45,266, Joseph G. Contrera, Registration No. 44,628, Chalin A. Smith, Registration No. 41,569, Shelly Guest Cermak, Registration No. 39,571, Patrick Stanzione, Registration No. 40,434, Suzannah Sundby, Registration No. 43,172 of SHANKS & HERBERT, telephone (703) 683-3600, with a mailing address at:

TransPotomac Plaza
1033 N. Fairfax St., Suite 306
Alexandria, VA 22314

with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith.

The undersigned hereby authorizes the U.S. Attorneys named herein to accept and follow instructions from undersigned assignee, if any, and/or, if the undersigned is not a resident of the United States, the undersigneds domestic attorney, patent attorney or patent agent, as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and the undersigned. In the event of a change in the person(s) from whom instructions may be taken, the U.S. attorneys named herein will be so notified by the undersigned.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issued thereon.

Full Name of Sole/First Inventor: Hyeyoung LEE

Inventors Signature: Hyeyoung Lee Date: Oct. 21, 2000
Residence: Seoul Citizenship: Republic of Korea
(City, State) (Country)
Post Office Address: No. 190-1106, Woosung APT., Yangjae-1-dong, Seocho-ku, Seoul,
Republic of Korea

Full Name of Second/Joint Inventor: Young Kil PARK

Inventors Signature: Young Kil Park Date: Oct. 21, 2000
Residence: Seongnam-si, Kyunggi-do Citizenship: Republic of Korea
(City, State) (Country)
Post Office Address: 534-1, Seongnam-dong, Joonwon-ku, Seongnam-si, Kyunggi-do,
Republic of Korea

Full Name of Third/Joint Inventor: Gill-Han BAI

Inventors Signature: Bai Gill Han Date: Oct. 21 / 2000
Residence: Seongnam-si, Kyunggi-do Citizenship: Republic of Korea
(City, State) (Country)
Post Office Address: No. 129-805 Parktown, Naejeong-dong, Boondang-ku, Seongnam-si, Kyunggi-do,
Republic of Korea

Full Name of Fourth/Joint Inventor: Sang-Jae KIM

Inventors Signature: Sj Kim Date: Oct. 21, 2000
Residence: Seoul Citizenship: Republic of Korea
(City, State) (Country)
Post Office Address: No. 310-103, Seonsoochon APT., 89, Banglee-dong, Songpa-ku, Seoul,
Republic of Korea

Full Name of Fifth/Joint Inventor: Sang-Nae CHO

Inventors Signature: Sangnae Cho Date: Oct. 21, 2000
Residence: Seoul Citizenship: Republic of Korea
(City, State) (Country)
Post Office Address: No. 111-602, Hansin ATP., 929, Mok-6-dong, Yangchon-ku, Seoul
Republic of Korea

Full Name of Sixth/Joint Inventor: Yeun KIM

Inventors Signature: Yeun Kim Date: Oct. 21. 2000
Residence: Pajoo-si, Kyunggi-do Citizenship: Republic of Korea
(City, State) (Country)
Post Office Address: 56-10, Moonsan-ri, Moonsan-eup, Pajoo-si, Kyunggi-do,
Republic of Korea

Full Name of Seventh/Joint Inventor: Hee Jung PARK

Inventors Signature: Park Heejung Date: Oct. 21. 2000.
Residence: Seoul Citizenship: Republic of Korea
(City, State) (Country)
Post Office Address: No. 102-209, Dongjin-villa, 341, Chang-1-dong, Dobong-ku, Seoul,
Republic of Korea

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: LEE, et al.

Serial No.: To Be Assigned

Filed: Herewith

For: rpoB Gene Fragments and a Method for the
Diagnosis and Identification of Mycobacterium
Tuberculosis Mycobacterial Strains

Art Unit: To Be Assigned

Examiner: To Be Assigned

Atty. Docket: 0217-0004

**SUBMISSION OF SEQUENCE LISTING
UNDER 37 C.F.R. § 1.821(a)**

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

Sir:

In compliance with 37 C.F.R. § 1.821(a), Applicants submit the Sequence Listing,
including the paper copy of the Sequence Listing and the computer readable copy of the
Sequence Listing.

In the Specification:

Please enter the Sequence Listing between the specification and the claims of the
above-identified application.

REMARKS

In accordance with 37 C.F.R. § 1.821(f), the paper copy of the Sequence Listing and the computer readable copy of the Sequence Listing submitted herewith in the above application are the same.

In accordance with 37 C.F.R. § 1.821(g), this submission includes no new matter.

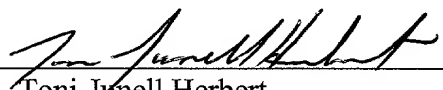
It is respectfully believed this application is now in condition for allowance.

Early notice to this effect is earnestly solicited.

Respectfully submitted,

SHANKS & HERBERT

By:



Toni-Junell Herbert

Reg. No. 34,348

Date: 10/26/00

TransPotomac Plaza
1033 N. Fairfax Street
Suite 306
Alexandria, VA 22314
(703) 683-3600

SEQUENCE LISTING

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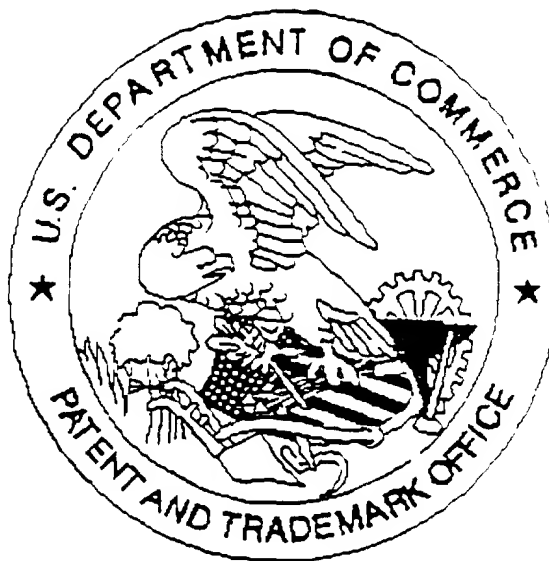
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Drawings